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Current reproductive toxicity tests may be missing significant toxicity by not examining the gametes of female organisms. The purpose of the present study is to develop and validate a reproductive toxicity test using the gametes and embryos of *Xenopus laevis*. Sexually mature female *Xenopus laevis* were exposed to varying concentrations of cadmium chloride ($CdCl_2$) in order to examine the effects of cadmium (Cd) upon oogenesis and progeny. Frogs were exposed for a period of 21 days and then either bred to untreated males or their ovaries were examined to determine effects upon oogenesis. Preliminary evaluation of the protocol for reproductive assessment appear favorable. Results of $CdCl_2$ exposure indicated that Cd significantly disrupted the process of oogenesis. The percentage of ova were reduced at most stages, there was a significant increase in atretic oocytes, and ovaries readily accumulated Cd via subcutaneous injection of Cd into the dorsal lymph sac of females. Additionally, results indicated that Cd is transferred maternally and that the FETAX assay can be easily modified to assess the effects of maternal exposure to reproductive toxicants upon progeny. The present study provides the protocol for a reproductive toxicity assay

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FOREWORD

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John A. Beatle _____ 10/14/98
PI - Signature   Date

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Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for Care and Use of Laboratory Animals (NRC 1996) in facilities that are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International.

INTRODUCTION

A. OBJECTIVE

The purpose of this project is to develop and validate a reproductive toxicity test using the gametes and embryos of the South African Clawed frog, *Xenopus laevis*. The use of *Xenopus* provides an alternate species to standard mammalian tests and uses the same species as the Frog Embryo Teratogenesis Assay - *Xenopus* (FETAX). The specific objectives of this project are:

1) Develop and evaluate exposure methods that effectively deliver toxicants to adult female frogs. Four experimental exposure methods will be evaluated. The first method is direct exposure to an experimental substrate (dermal), the second method is direct uptake of the toxicant by ingestion in food (oral), the third method is by intraperitoneal (i.p.) injection (parenteral), and the third method is by subcutaneous (s.c.) injection into the dorsal lymph sac. Optimal exposure times will also be determined.

2) Identify and evaluate endpoints of a reproductive toxicity test that will assess the toxicity of contaminants on oocyte growth, development and ability to fertilize as well as test the effect of the toxicants on the ability of the treated frog oocytes to develop normally following ovulation and fertilization. The most sensitive, cost-effective and repeatable reproductive toxicity endpoints will be selected.

3) Develop a preliminary protocol and validate it using three reproductive toxicants.

4) Rewrite the protocol based on the results obtained with the first three compounds and then test the first three over again along with four additional compounds.

5) Submit the final protocol to the American Society for Testing and Material (ASTM) for approval.

B. SIGNIFICANCE OF THE CURRENT RESEARCH

Cellular and molecular processes differ among the various stages of an organism's life cycle. These differences can cause a varied sensitivity to xenobiotics, where some life stages may be more sensitive because of specific cell receptors or differences in metabolism. Therefore, it is important that toxicity screening assays account for the different stages of the life cycle, especially the most sensitive stages such as within reproduction and development. Reproduction and development are complex and specialized processes that begin with gametogenesis, continue

through fertilization and embryogenesis, and end in the sexual maturation of the organism. In designing a reproductive toxicity assay, it is critical to assess toxicity at the stages in an animal's life cycle when sensitivity to toxicants occur (Mattison and Thomford, 1989). For example, gametes can be affected by toxicants at levels well below those of adults. Many current, short term, reproductive and developmental toxicity tests may be missing significant toxic events by excluding the effects on gametes and full life cycle studies are not practical in most vertebrates due to their long life spans. It may be wiser to use a battery of short-term tests to assess toxicity to vertebrates. It is more economically feasible to use the same species for as many tests as possible. Therefore, the reproductive toxicity test we are developing complement the more developed FETAX developmental toxicity test and a new thyroid test being developed using *Xenopus* (D.J. Fort and J.A. Bantle). In order to achieve a thorough assessment of hazards to an organism, toxicity testing must take into account the different stages in the organism's life cycle. Testing should, therefore, incorporate all aspects of toxicity including immunotoxicity, neurotoxicity, developmental toxicity, and reproductive toxicity. Reproductive toxicity tests should examine the effects of xenobiotics on gametes, fertilization, and progeny.

Historically, much of reproductive toxicity testing had centered primarily on the male reproductive system. Concern with male reproduction has escalated following the discovery of declining sperm counts over the past 50+ years by some investigators, although this is controversial. Research has been conducted on the processes of spermatogenesis as well as changes in testes morphology (Mori et al., 1992; Majumder and Kumar, 1995; Llobet et al., 1995; Pant et al., 1995; Boockfor and Blake, 1997). Female reproductive toxicity was not vigorously studied until recently and as such there are very few validated assays that test toxicant effects on the female reproductive, embryonic and developmental cycles (Kamrin et al., 1994). The preponderance of female reproductive toxicity tests are conducted with mammals, specifically on events occurring after fertilization (Levin and Miller, 1980; Rice, 1981; Wier et al., 1990; De et al., 1993). For example, researchers have examined the effects of toxicants on post-fertilization events such as the implantation of embryos (Rice, 1981; Eisenmann and Miller, 1994) and interaction between mother, placenta, and fetus (Wier et al., 1990). Additionally, the cellular processes such as gap junction communication during muscular contractions in the uterus have been reviewed by Kamrin et al. (1994). Paksy et al. (1997) showed that Cd interfered will cell-cell junctions and cell adherence in rats. Studies have also been conducted

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with vertebrates other than mammals. Michibata et al. (1987) have examined the survivability of teleost fish eggs exposed to varying toxicant concentrations, the amount of toxicant accumulated by the egg, and the location of the toxicant within the egg and surrounding membranes.

The endpoints that can be evaluated in a reproductive toxicity test are numerous. Adult animals can be exposed and their gonads and/or gametes examined for morphological changes (Lohiya, 1976; Mattison et al., 1983; Sato and Koide, 1984; Jarrell et al., 1988; Toth et al., 1992; Domingo, 1995). The effect of the toxicant on fecundity can be examined by exposing adult females (Kramer et al., 1998). Transgenerational effects on the progeny of exposed adults can be assayed using the endpoints from FETAX. FETAX endpoints include mortality, malformation, growth inhibition, behavior, pigmentation, locomotion and feeding ability. Additionally, a 96-h LC50 (mortality) and 96-h EC50 (malformation) can be determined by probit analysis of the data collected from FETAX. A teratogenic index (TI) can then be calculated by dividing the LC50 by the EC50. The TI is a comparison of observed toxicities between an adult and embryo and is a measure of developmental hazard. A compound with a TI < 1.5 is interpreted to have a low teratogenic hazard whereas higher values have signified an increase in the potential teratogenic hazard.

Amphibians represent an ideal model of vertebrate development (Muller, 1997) and *Xenopus laevis* is well suited for female reproductive studies because of its fecundity and continuous asynchronous oogenesis. A new reproductive cycle can be initiated *in vitro* during any season by administration of hormones (Dumont, 1972). Additionally, an extensive knowledge base has been developed through its use for research in developmental and molecular biology. The study of *Xenopus* oogenesis has led to our understanding of the molecular mechanisms of human oogenesis (Muller, 1997).

C. REPRODUCTIVE TOXICITY ASSAY DESIGN

Gametogenesis and fertilization are highly specialized processes. Meiosis is the reduction of chromosomes from a diploid to a haploid number and takes place only during gametogenesis. Interruption of meiosis can result in aneuploidy or polyploidy as well as other chromosomal defects of a hazardous nature.

At the onset of amphibian oogenesis, the oocytes begin meiosis but are arrested in the diplotene stage of prophase I. During the diplotene stage, the chromosomes become highly

extended into a lampbrush configuration and are very active in RNA synthesis (Gilbert and Raunio, 1997). These specialized chromosomes direct the metabolic activities of the developing cell. Along with the lampbrush chromosomes, gene amplification also occurs during this stage (Gilbert and Raunio, 1997). During gene amplification ribosomal RNA (rRNA) is replicated for the synthesis of ribosomes, which is necessary for synthesizing proteins. Proteins are needed to supply the growing oocyte as well as carry the embryo through the first stages of development.

Diplotene is also a period of oocyte growth and differentiation. The oocytes grow mainly by accumulating yolk through vitellogenesis. Vitellogenin may be an important route of exposure to toxicants, functioning as a carrier protein for inorganic phosphates, lipids, carbohydrates, and metals that ultimately get incorporated into the oocyte (Ghosh and Thomas, 1995). Once incorporated into the oocyte, vitellogenin is converted into yolk proteins (Wallace and Bergink, 1974). A majority of the yolk accumulates in the vegetal hemisphere of the growing oocyte. This hemisphere is yellowish in color and contains mRNAs essential for organism development. The other half of the oocyte, the animal hemisphere, is darker in pigmentation and contains the nucleus, or germinal vesicle. During development, most of the organs come from the animal hemisphere (Gilbert and Raunio, 1997). The end product of oogenesis, an oocyte, is a highly polarized cell. This cell contains enough material to form a complete embryo in the absence of sperm (Gilbert and Raunio, 1997). Additionally, the oocyte contains numerous morphological and physiological adaptations that permit rapid and controlled embryonic development.

Oocyte maturation begins when luteinizing hormone (LH) stimulates the follicle cells around the oocyte to produce progesterone (Gilbert and Raunio, 1997). Upon oocyte maturation, meiosis resumes and the germinal vesicle breaks down (GVBD). During GVBD microvilli retract, nucleoli disintegrate and the lampbrush chromosomes contract. The oocyte divides and becomes arrested for a second time in metaphase II. The course of *Xenopus* oocyte maturation and the associated biochemical changes have been extensively researched (Sato and Koide, 1984; Cicirelli and Smith, 1985; Taylor and Smith, 1987). Cicirelli and Smith (1985) found that levels of cyclic adenosine monophosphate (cAMP) declined upon oocyte maturation. Exposure to toxicants that interfere with the decline in cAMP levels cause the oocyte to remain in meiotic arrest thereby impeding maturation. Fertilization is the next signal that allows completion of meiosis and the subsequent fusion of pronuclei. The new embryo utilizes its store of histones, energy, mRNA, and proteins to proceed rapidly through development. Missing genetic

information or the effect of the toxicant on early development will be quickly translated into malformed, stunted or dead embryos

D. PRELIMINARY OR RELATED STUDIES

1. Reproductive toxicity testing research

Previous work was conducted in this laboratory in which the reproductive toxicity of JP-4 contaminated soil was examined using *Xenopus*. Tests were conducted with known reproductive toxicants to evaluate endpoint selection and then the reproductive toxicity of the contaminated soil was examined. Adults were exposed either orally or directly (dermally) for a period of 60 days. Although the direct exposure method was successful in this study we concluded that direct i.p. or s.c. injection should be explored in the future. Additionally, attempts should be made to shorten the length of exposure. More research with positive controls was recommended. Analysis of oocytes *in vitro* proved to be a valuable endpoint, however, we concluded that two groups of female frogs should be used, one for oocyte analysis and the other for breeding.

2. Development of FETAX

The original work on developing a developmental toxicity screening assay using *Xenopus* embryos to detect environmental teratogens was performed in the laboratories of Greenhouse (1978) and Dumont (1983). Greenhouse used 48-hour exposures to military compounds to demonstrate toxic and teratogenic effects on developing embryos. Early studies in our laboratory have demonstrated that FETAX can be used with a variety of chemicals and complex mixtures. The endpoints include: LC50 (mortality), EC50 (malformation-teratogenesis), no observed effect concentration (NOEC), minimum concentration to inhibit growth (MCIG), motor behavior, pigmentation, and gross anatomy. The assessment of teratogenic potential has been based on TI values, embryo growth, and severity and types of induced malformations. TI values <1.5 have a greater possibility of embryos being malformed in the absence of significant embryo lethality. Types and severity of induced malformations have also been considered, especially for compounds with TI values <1.5 which produce serious defects of major organ system. Such compounds may still pose a serious threat, possibly as embryotoxins. Test chemical exposures have been continuous for 96-hours and mortality and stage of development were checked every 24 hours while other endpoints were recorded only at 96 hours. Test compound renewal was

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performed daily. Data collection was simple, as all observations were made using a dissection microscope. The data collection using FETAX have been in harmony with the criteria for an *in vitro* teratogenesis screen suggested by Kimmel et al. (1982) which include: good concentration-response relationships, adequate number of embryos, and easily defined and quantifiable endpoints.

With over 100 compounds tested we have approached 95% predictive accuracy using FETAX with the *in vitro* metabolic activation system (MAS) developed for FETAX. We are currently performing an interlaboratory validation study with several laboratories. Results obtained to date have been extremely encouraging and warrant further study. Addition of FETAX endpoints to the reproductive toxicity assay further the utility of both tests.

BODY

A. EXPERIMENTAL METHODS

1. Evaluation of exposure methods

Our first objective was to determine the most suitable exposure regimes in which toxicants could be administered to the adult male and female frogs. Of the exposure routes possible, the following regimes were considered: per os, s.c. via dorsal lymph sac, i.p., and dermal/oral via the culture water. Experiments with boric acid and cadmium have been performed. These compounds were selected because of ease of analytical measurement of bioaccumulation in the reproductive organs. Oral administration of boric acid was provided in the form of supplemented rat chow (ca. 1,600 mg/Kg) which was fed to the frogs on a daily basis for 28-d. Boric acid (100 mg B/Kg/day) in amphibian saline was also injected s.c. into the dorsal lymph sac and i.p. into the body cavity in different sets of frogs daily for five days and the ovaries and testes removed for boron analysis. Control treatments included frogs injected with amphibian saline alone, frogs fed a standard rat chow diet, and frogs fed beef liver and lung (ASTM diet). No dermal (water borne) application was attempted with boric acid.

Similar experiments were conducted with cadmium with the only exception being that earthworms were used as the food source for per os administration with cadmium being injected directly into the worms which were then fed to the adult frogs. The parenteral dose was 2.5 mg/Kg/day of cadmium for five successive days. Also, exposure via the culture water was evaluated by exposing the adult frogs to 2.5 mg/L Cd for 5 days with daily renewal. Controls for each treatment were utilized. For parenteral routes of administration saline was used. For oral routes, unspiked food was used. Boron and cadmium are being measured by ICP-MS and GF-AAS, respectively.

Secondly, the protocols developed for assessing reproductive toxicity in the female frogs using ovary maturation status as a marker was used in our lab to assess the effects of boric acid. These procedures as outlined by Dr. Bantle's laboratory were used to determine both toxic effects and the effects of nutritional deficiency. We also used the methods developed by Dr. Bantle's laboratory, as well as those developed at USACEHR to evaluate impact on the male frogs.

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2. Reproductive toxicity endpoint evaluation and protocol development

Mature female and male *Xenopus* were obtained from Xenopus I (Dexter, MI) and allowed to acclimate for a period of 14 days before being number branded with liquid nitrogen for identification. Each experiment was composed of one control group and two treatment groups; each group contained the same number of frogs. Frogs were randomly divided into treatment groups and housed them in six identical heavy plastic laundry tubs containing 15 L of filtered, non-chlorinated water. No more than 4 frogs were housed per tub. The water was changed three times weekly; temperature was kept between 20 and 22° C and monitored daily. Frogs were housed in a room with a photoperiod of 12h light: 12h dark. They were fed three times per week a diet of ground beef liver and lung containing vitamins. Frogs were weighed weekly and behavior was monitored routinely.

Frogs were injected s.c. via the dorsal lymph sac every other day for 21 days with either 0.7% saline or Cd as Cadmium Chloride ($CdCl_2$) (Aldrich Chemical CAS #10108-64-2) in saline solution at the dose levels (mg/kg body weight). Frogs in experiments 1 (3.0 and 5.0 mg/kg) and 2 (0.5 and 5.0mg/kg) were injected with Cd immediately following the acclimation period. Frogs in experiments 4 (0.5 and 3.0 mg/kg), 5 and 6 (0.75 and 1.0 mg/kg) were induced to ovulate 20 days prior to beginning Cd exposure by injection with 800 IU Human Chorionic Gonadotropin (hCG) (Sigma #CG-5).

a. Cd Effect on Oogenesis

After the final injection, frogs were anesthetized with MS-222 until unresponsive and then killed by cervical dislocation, the ovaries, livers and spleens were immediately removed and weighed. Livers and spleens were stored in 3% formalin while ovaries were placed in Barth's medium (Smith et al., 1991). The organ weight to body weight ratio for ovaries, livers, and spleens was calculated and the data analyzed for non-stimulated and stimulated frogs. Necropsies were performed noting any gross lesions or tumors. A lobe from the ovary was removed. Ova were isolated according to Smith et al. (1991), staged by diameter (using an ocular micrometer) and morphology as per Dumont (1972) and stored in 3% formalin. Ova at each stage of oogenesis were compared between control frogs and treated frogs.

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b. Cd Effect on Progeny

Treated and control females were mated to untreated males. Animals were induced to breed by injection with hCG (800 IU - females, 400 IU - males). Mating behavior was observed and deviations from normal recorded. Following successful mating, 200 eggs from each pair were double sorted according to standard ASTM FETAX (Bantle et al., 1991) procedures and allowed to grow for 96 hours in an incubator at 24° C. After 4 days (Stage 46), mortality of the embryos was assessed, embryos were anesthetized in 4% MS-222 and fixed in 3% formalin. Malformation and length data were gathered as in standard FETAX protocol.

c. Determination of Cd Concentration

For analysis of Cd in ovaries, eggs and embryos, samples were homogenized and acidified with trace-metal grade nitric acid (HNO_3). Samples and blanks were sent to the U.S. Army Center for Environmental Health Research (USACEHR) for Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) analysis. Techniques for sample processing and microwave digestion were conducted to maximize recovery and to minimize extraneous metal contamination using Environmental Protection Agency Method 6020. Once the samples arrived at Ft. Detrick they were transferred to acid washed Teflon digestion vessels. Additional HNO_3 was added to make the final volume 10 ml. Samples were digested by microwave using the CEM Microwave Sample Preparation System (CEM Corp., Matthew, NC). Following digestion, the samples were diluted to 40 ml with distilled water and filtered through a 0.45 μm Teflon membrane. Cadmium standards were prepared at 0, 1, 10 and 100 μg/L. An internal standard of Yttrium 89 at 1 ppm was added prior to nebulization. Following standardization the calibration curve was verified by analyzing a 100 μg/L calibration standard and a blank. The check standard was repeated at a 10% frequency during the analysis and after the analysis was completed. The acceptable range for the check standard was +/- 10% of the actual value.

Analyses were performed on all samples using a Hewlett Packard Model 4500 Inductively Coupled Plasma Mass Spectrometer. Final Cd concentrations were corrected for internal standard recovery, analysis dilution, digestion volume and the original mass of the sample. The lower limit of sensitivity was 0.1 μg/g wet weight of the samples.

d. Statistical analysis

All data were initially tested for normality and homogeneity of variance. To determine differences in oogenesis and organ-to-body weight ratios between control and treated frogs, either a one-way ANOVA or Kruskal-Wallis one way ANOVA on Ranks, depending on normality, was performed (Zar, 1984). Multiple comparison procedures were performed using either Dunnett's or Dunn's Method (Zar, 1984). The data were analyzed using a two-way ANOVA to evaluate time of experiment and exposure effects (Zar, 1984). Analysis of embryo mortality and malformation rates was performed using the Chi-Square test of equal proportions and Bonferroni confidence interval procedure. The Student's t-test was used to examine the differences in embryo length between the exposure groups (Zar, 1984). The data from the 0.5 mg/kg exposure groups was deleted for mortality, malformation, and length analysis due to the small sample size (n = 1).

Concentrations for ICP-MS were reported as $\mu g/g$ wet weight. Concentrations below the detection limits of the ICP-MS were represented as zero for statistical analysis. A one-way ANOVA and Dunnett's test were employed to determine differences between control and treated animals (Zar, 1984).

All statistical analysis were performed at the $p < 0.05$ level of significance. ANOVAs and multiple comparison procedures were performed with the SigmaStat software from SPSS Science, Chicago, IL. The Student's t-test, Chi-square test, and Bonferroni procedure were performed using the Statistical Analysis Software from SAS Institute, Cary, NC.

B. RESULTS

1. Evaluation of exposure methods

Results of boric acid administration studies are presented in Tables 1 and 2. Results from the exposure studies with boric acid indicated that the most effective routes of exposure were parenteral, specifically s.c. injection via the dorsal lymph sac based on the proportion of the dose administered found in either the ovaries or testes (Table 2). However, oral administration via the food worked quite well with a 28-d exposure period. Elevated levels of boron were detected in

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both the ovaries and the testes. In addition, dermal adsorption of boron was greater than expected.

Results of cadmium administration studies are presented in Tables 3 and 4. With cadmium, the most effective routes were parenteral administration (i.p. and s.c. via the lymph sac) in either species. Oral administration via laced worms and dermal exposure via the culture water were much less effective (Table 3). Overall, the females accumulated cadmium more effectively than the males for each of the routes used in this study (Table 4). Nearly 50% of the dose administered was retained within either the ovaries or the oviducts via parenteral routes. The males retained nearly 30% of the dose administered via parenteral routes.

In our initial studies, which are slated to be repeated, we found that boric acid supplied via the food (2,000 mg/Kg) for 28-d caused effects in both male and female frogs. Effects in the female were characterized as abnormal ovary morphology and a reduction in mature oocytes compared to the controls. In the males, we observed a reduction in sperm count, a reduction in motility, and slight (statistically significant) increase in sperm dysmorphology. It is questionable, however, whether this effect is biologically significant since the fertilization rates remain fairly high.

2. Endpoint and protocol evaluation

   a. Oogenesis - Non-stimulated Frogs

Cadmium had a significant effect on oogenesis in non-stimulated and stimulated frogs (Figures 1 - 4). In non-stimulated frogs there was a concentration-dependent decrease in the percentage of stage II oocytes (Figure 1). This decrease was significant at 3.0 and 5.0 mg/kg ($p < 0.0001$). There was a decrease in stage III oocytes from frogs exposed at 5.0 mg/kg ($p < 0.0001$) while a slight increase in stage III oocytes at 0.5 mg/kg frogs was exhibited ($p > 0.05$). All exposure groups exhibited a decrease in the percentage of stage IV oocytes ($p < 0.0001$). Although the percentage of stage V oocytes increased at all exposures only at 5.0 mg/kg was the increase significant ($p = 0.0013$). The percentage of stage VI oocytes in all exposure groups was different from controls ($p < 0.0001$). Furthermore, there was an increase in atretic eggs in all exposure groups, however, only at 3.0 and 5.0 mg/kg was the increase significant ($p < 0.0001$).

Estimation of the total number of oocytes/g ovary at each stage (Figure 2) revealed the same trends and statistical differences as in the percentage data (Figure 1) for stages II, III, IV and

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atretic oocytes. Statistical examination of the total number of oocytes revealed no significant difference in the number of stage V oocytes at all exposures while the number of stage VI oocytes decreased in the 3.0 and 5.0 mg/kg exposure groups ($p < 0.0001$).

b. Oogenesis-Stimulated Frogs

Cadmium also affected oogenesis in hCG-stimulated frogs (Figures 3 and 4). There was a decrease in the percentage of stage II oocytes in the 3.0 mg/kg exposure group ($p = 0.04$) (Figure 3). The percentage of stage III oocytes was also decreased. The decrease was significant at 0.75 mg and 3.0 mg/kg ($p = 0.007$). There was a significant difference in the percentage of stage IV oocytes between the exposure groups ($p < 0.001$) but not between control and exposed frogs. The percentage of oocytes in the 0.75 and 1.0 mg/kg groups increased over the control while there was a decrease in the 0.5 and 3.0 mg/kg groups. The percentage of stage V oocytes at 0.5 mg and 3.0 mg/kg were decreased ($p = 0.03$). Statistical analysis of the stage VI oocytes revealed a significant difference ($p = 0.006$), however because very few stage VI oocytes were produced during the exposure period, it was difficult to make comparisons between control and experimental groups. There was a significant increase in the percentage of atretic oocytes in all exposure groups ($p < 0.0001$). Atretic oocytes at the lowest concentration exhibited a mottled and swirled appearance, while at 3.0mg/kg, the ovaries of most frogs contained mostly completely degenerated oocyte follicles (Figure 6).

Estimation of the total number of oocytes/g ovary revealed basically the same trends in all stages across all exposure groups (Figure 4). There was a significant decrease in stage II oocytes at 3.0 mg/kg ($p = 0.04$). However, the decrease was not significant in stage III oocytes ($p = 0.06$). In stage IV oocytes, there was a difference between the 1.0 mg/kg and 0.5 and 3.0 mg/kg exposure groups only ($p = 0.0001$). There was no significant difference in the number of stage V oocytes although there is a distinct decrease in the population of oocytes. There was a decrease in stage VI oocytes ($p = 0.03$), however due to the high incidence of zeros in the data set a multiple comparison test was not valid. All exposure groups exhibited a significant increase in atretic oocytes ($p = 0.0004$).

c. Cd effect on progeny

The percentage of successful matings between Cd-exposed females and non-exposed males was significantly decreased from control frogs (p = 0.02). In four experiments, 12 out of 22 (54.5%) control animals mated successfully, 1/9 (11%) at 0.5 mg/kg, 4/14 (28.6%) at 0.75 mg and 1.0 mg/kg, and 0/8 (0%) animals mated successfully at 3.0 mg/kg. The results of the modified FETAX test on the progeny from females that did mate successfully are shown in Table 5. Because the percentage of successful matings was extremely low at 0.5 mg/kg (1 out of 9) and zero at 3.0 mg/kg statistical analysis of the mortality, malformations, and growth was limited to the control, 0.75 mg and 1.0 mg/kg groups. Mortality and malformations appeared to decrease with increasing Cd concentration. The Chi-square test indicates that the probability of mortality is dependent on the level of exposure ($\chi^2_2 = 252.1289$, p < 0.001). The Bonferroni confidence interval procedure shows that the 0.75 mg/kg exposure group is statistically different than the control group. The probability of malformations also depended on the level of exposure ($\chi^2_2 = 44.4026$, p < 0.001), and once again the difference lies between the control and 0.75 mg/kg exposure group. Statistical analysis of the head-to-tail length data revealed no significant difference between progeny from exposed females and controls (p = 0.60).

In malformed progeny from control females, edema was observed most frequently (66.7%) with gut malformation being the second most frequent malformation (40%). The most common malformations in embryos from Cd-exposed females, including the 0.5 mg/kg group, are shown in Figure 5. The highest incidence of malformations in the 0.75 and 1.0 mg/kg groups was in the notochord (61.3 % and 61.9%, respectively). Notochord malformations mainly occurred at the proximal end causing the body axis of the embryo to curve either vertically or horizontally (Figure 7). Because the tails were malformed, embryos swam abnormally, i.e. in circles, after 72 h. Embryos with and without notochord deformities from females exposed at 0.75 mg/kg were allowed to grow past 92 h. All embryos with notochord deformities died within 116 h post-fertilization while the non-malformed embryos from the same exposure group survived beyond 140 h. Cadmium also increased the incidence of gut malformations. In the 0.75 mg/kg group 38.7% of embryos and 57.1% of embryos from the 1.0 mg/kg group exhibited loosely coiled guts (Figure 8). Eye deformities were also observed which included misshapen lenses and abnormal eye placement. Other malformations observed included misshapen and shortened heads and deformed mouths.

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d. Cd content in ovaries and embryos

ICP-MS analysis was conducted to examine the Cd concentration in frog ovaries in both stimulated and non-stimulated frogs. Table 6 shows the ovary Cd concentrations in non-stimulated and stimulated frogs and also the percentage of Cd injected that was accumulated by the ovary. The Cd concentration in the ovaries of non-stimulated frogs was significantly different from control frogs at the 5.0 mg/kg exposure level ($p = 0.004$). In stimulated frogs, there was a difference at the 0.5 mg and 3.0 mg/kg levels ($p < 0.0001$). Cadmium concentrations within the ovaries ranged from 2.29 to 15.08 μg/g wet. The percentage of Cd injected that was accumulated by the ovary appeared to decrease with increasing Cd dosage. Cadmium was readily detected by ICP-MS analysis in progeny of Cd-exposed females (Table 7). Statistical analysis of these preliminary results indicated no significant difference in fertilized egg or embryo Cd concentrations between control and exposed frogs. The data indicated that there was a greater concentration of Cd in embryos at 24 h than in fertilized eggs. The data reveal that the embryo Cd concentration decreased over the length of the FETAX assay, based on measurements at 24, 48, 72, and 96 h (i.e. stages, 26, 37, 42, and 46, respectively) (Nieuwkoop and Faber, 1967). Although the decrease was not statistically significant, the small sample size caused the variances to be extremely high.

e. Cd effect on organ weight

Analysis of the ratio of the organ weight to body weight of the liver, spleen and ovary indicated that in non-stimulated frogs there was a significant increase in the spleen-to-body weight ratio in the 0.5 and 3.0 mg/kg groups ($p < 0.0001$) (Table 8). Statistical analysis revealed no differences in the ratios for the liver, spleen, or ovary in stimulated frogs. Frogs exposed at 3.0 and 5.0 mg/kg exhibited a higher incidence of edema due to large quantities of yellowish fluid within the body cavity.

CONCLUSIONS

Preliminary results suggest that the most effective routes of exposure are parenteral. Dermal exposure (cadmium only) was the least effective and oral administration (females only) was only slightly better. These results are not necessarily unexpected because the majority of cadmium consumed via enteral routes will be cleared quickly from the body and thus, not absorbed into the body. Results from these studies indicated that we can expect differences in accumulation based on sex and the toxicant being studied.

An additional consideration is the relevance of each route of exposure. Feeding represents a more reasonable exposure route that parenteral administration in the environment. However, feeding studies require longer periods of exposure time and are more difficult to control in terms of external variables. Injection into the lymph sac is technically more simplistic and can be accomplished over a shorter period of time. Although it is not necessarily relevant as an environmental exposure route, it is a commonly used route in toxicological studies. Therefore, although all studies are not yet complete, it would appear that if environmental realism is not a concern, parenteral administration should be considered the primary means of toxicant administration. In circumstances where environmental realism is an issue, oral administration should be used.

Our study found that the stages of oogenesis are a sensitive and relative endpoint in a reproductive toxicity assay. Dumont (1972) correlated the stages of oocyte development in *Xenopus* with the related physiological and biochemical data. By examining thousands of oocytes at each developmental stage Dumont (1972) determined the percentage of oocytes at each stage of oogenesis in non-stimulated frogs. The oogenesis data with control non-stimulated frogs in the present study are consistent with Dumont's findings.

At the onset of this study female *Xenopus* were exposed to Cd immediately following the acclimation period. These animals/experiments are referred to as non-stimulated. Dumont (1972) suggested that oocytes from non-stimulated frogs were not as metabolically active as those from females stimulated with hCG. Our results indicate that the effect of Cd upon oogenesis is more severe in hCG-stimulated frogs. When hCG is administered to female *Xenopus,* they ovulate most of their mature oocytes and begin a period of vitellogenesis (Dumont, 1972). Therefore, hCG promotes an increase in protein incorporation (Wallace et al.,

1970). Keem, et al. (1979) measured the growth rate of oocytes in laboratory-maintained *Xenopus*. They noted that even when all environmental conditions were controlled, there still existed a large variability in the patterns of oocyte growth between non-stimulated and hCG-stimulated frogs. It took 16-24 weeks for oocytes from non-stimulated frogs to progress from stage III to stage VI while in hCG-stimulated frogs the time period for the same growth was 9-12 weeks. The greatest amount of oocyte growth occurred 20 to 42 days after hCG-stimulation (Keem et al., 1979). In order to standardize reproductive status, decrease variability, and to achieve the greatest uptake of Cd by the ovaries while keeping the length of the toxicity assay as short as possible, it was decided that hCG-stimulated frogs would be used in the rest of the present study. Furthermore, to target the critical growth phase, exposure to Cd was begun 20 days after hCG-stimulation and the frogs were exposed for 21 days.

Ovaries from control frogs in the present study contained oocytes at all stages and most oocytes appeared healthy. For example, stage IV - VI oocytes had distinct animal and vegetal hemispheres and relatively few atretic oocytes were present. There was, however, a significant increase in the population of atretic oocytes in Cd-exposed frogs. In both non-stimulated and hCG-stimulated frogs, and at all concentrations of Cd tested, the ovaries contained a large portion of atretic oocytes. The morphology of these oocytes was consistent with that described by Dumont (1972). A high incidence of small, very darkly colored, spheres present only in the theca were common in the present study. Dumont (1972) categorized these as completely degenerated oocyte follicles containing densely packed pigment. Our findings are consistent with those of Pramoda and Saidapur (1986) in which *Rana tigerina* were exposed to 600 µg $CdCl_2$ via i.p. injection. After 30 days they performed a histological examination of the ovaries. They found that Cd severely affected the vitellogenic growth of oocytes and increased the number of atretic oocytes. They hypothesized that Cd affected vitellogenic growth by compromising the function of the liver, altering metabolism, and perhaps reducing the blood and nutrient supply to the follicles. In starvation studies with *Xenopus*, Dumont (1972) noted that only oocytes containing yolk were susceptible to atresia. Although no visibly degenerated stage II oocytes were found in this study, the population of stage II oocytes was decreased at every concentration (Figures 1-4) and the population of atretic oocytes was significantly increased. This suggests that stage II oocytes either underwent atresia or there was no recruitment from the

population of stage I oocytes. It is probable that Cd selectively removed oocytes from each stage of oogenesis, including stage II, thereby increasing the population of atretic oocytes.

The effects of Cd on oogenesis are much more apparent in hCG-stimulated frogs than non-stimulated frogs. As discussed by Dumont (1972), it appears that the ovaries of hCG-stimulated frogs in the present study have a higher metabolic activity and thereby are more actively involved in taking up Cd. Such a phenomenon also occurs when trypan blue is injected into hCG-stimulated and non-stimulated frogs (Dumont, 1972). Trypan blue is actively taken up by oocytes in hCG-stimulated frogs; however, the uptake is virtually nonexistent in non-stimulated frogs. The overall metabolic activity of oocytes from non-stimulated frogs is markedly diminished. Therefore, although the morphology of oocytes is the same at the identical stages in non-stimulated and hCG-stimulated frogs, there are submicroscopic cytological and physiological differences (Dumont, 1972).

The interaction of cadmium and vitellogenin synthesis has been the focus of many studies. Sunderman et al. (1995b) have shown that Cd binds to the vitellogenin yolk protein lipovitellin 1 in *Xenopus* and could be a mechanism for reproductive toxicity of Cd following environmental exposure. They hypothesized that Cd absorbed by the female could bind to plasma vitellogenin in place of zinc (Zn), enter oocytes by endocytosis and become deposited in the yolk platelets. Cadmium, when it is bound to vitellogenin, also becomes incorporated in the oocytes of Atlantic croakers (*Micropogonia undulatus*) (Ghosh and Thomas, 1995). Therefore, the binding of cadmium to vitellogenin could significantly increase the accumulation of the metal. Our data indicate that Cd is incorporated into stage IV and V *Xenopus* oocytes, and atretic oocytes of Cd-exposed females (results not shown). In further studies, we will compare the amount of Cd in oocytes at various stages.

Povlsen et al. (1990) discovered that one injection of 2 mg Cd/kg body weight caused a significant decrease in the amount of circulating plasma vitellogenin and calcium in the flounder [*Platichthyes flesus* (L.)]. Additionally, Cd exposure decreased the ratio of RNA to DNA in the liver. This indicates that Cd may interfere with transcription which, in turn, inhibits the vitellogenin synthesis. Transcriptional down-regulation of vitellogenin synthesis in response to Cd exposure has been reported by Olsson et al (1995) in the rainbow trout (*Oncorhynchus mykiss*). Because oocyte growth in fish and amphibians is through the uptake of vitellogenin, the

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inhibition of vitellogenin synthesis may lead to a decrease in oocyte number and/or delays in oocyte maturation.

Watanabe et al. (1977) performed chromosome analysis on ddY/F mice. The number of females with abnormal oocytes increased in the Cd-treated groups in conjunction with an increase in the number of oocytes exhibiting chromosome aberrations. They concluded that Cd has the potential to be a mutagen in mammalian meiotic chromosomes. Watanabe et al. (1977) also exposed mice to Cd via a single s.c. injection of $CdCl_2$ and then examined the oocytes and ovaries. They found a significant decrease in the number of oocytes recovered with increasing Cd concentration. We have shown that Cd caused a decrease in the oocyte population at most stages of oogenesis and at most concentrations of Cd tested (Figures 1-4, 6). Davidson (1976) suggests that the *Xenopus* ovary contains a reserve of oocytes in stage II from which groups are selected to undergo continued oogenesis. If this is true, then the decrease in the population of stage II oocytes from Cd-exposed frogs parlays into a decrease in oocytes in later stages and an overall decline in fecundity. This may permanently affect the ability of the female to reproduce.

Although Cd caused a decrease in the oocyte population in most stages of oogenesis and in most exposure groups, the population of stage V (non-stimulated frogs) and stage IV (stimulated frogs) oocytes at some exposures was greater than in the controls. These findings may be due to the variation in the number of oocytes present in any given stage prior to treatment in captive female *Xenopus* (Dumont, 1972). Physiological changes caused by Cd at stage IV could preclude further oocyte development to later stages. Interestingly, frogs exposed to Cd at a dose of 3.0 mg/kg did not breed (did not go into amplexus) and the percentage of frogs that bred at lower exposure concentrations was very low. Examination of the ovaries of the Cd-exposed frogs revealed that mature stage VI oocytes were present and, presumably, should have been ovulated. Frogs whose ovaries contained few or no stage VI oocytes did have stage V oocytes. Animals whose ovaries do not contain stage VI oocytes can still be induced to ovulate the most mature (stage V) oocytes upon hCG stimulation (Dumont, 1972). Therefore, Cd exposure may affect physiological or behavioral aspects of ovulation and breeding.

In dd/YF mice, Watanabe et al. (1977) found that the ovary Cd concentration increased relative to the dose. Additionally, the incidence of degenerated oocytes was higher in the treated groups. In the present study the concentration of Cd in the ovaries of Cd-exposed frogs was not positively correlated with dose. The ovaries of the lowest exposure group (0.5 mg/kg) exhibited

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a higher Cd concentration than the 0.75 and 1.0 mg/kg exposure groups. Additionally, the percentage of Cd that was accumulated by the ovaries decreased with increasing exposure concentration. It is possible that at the higher concentrations the ovary became overloaded with Cd causing necrosis of the ovary and the subsequent inability to accumulate any more toxicant. Figure 6 shows the dramatic loss of cells at higher Cd concentrations and illustrates that cell loss may well indicate why less Cd is present at high exposure doses. Alternatively, if vitellogenesis is the main mechanism by which the oocytes incorporate Cd and Cd inhibits vitellogenesis, then, at the higher exposure groups, vitellogenesis was inhibited causing a marked decrease in the uptake of Cd.

Maternal transfer of toxicants to offspring has numerous biological implications such as toxicity to progeny and transgenerational accumulation of contaminants (Standley et al., 1994). Possible mechanisms of maternal transfer in oviparous animals include incorporation into the lipids forming the egg mass, passive diffusion into mature eggs and active transport via vitellogenin. Some toxicants, such as organochlorines, are transferred from female to egg via lipids in mayflies (Standley et al., 1994) and in lake trout (Miller and Amrhein, 1995). Sato et al. (1996) found that transfer of Cd to eggs of leghorn chickens was restricted even when high amounts of Cd accumulated in the maternal liver. Cadmium accumulated in higher concentrations in the follicle walls rather than the follicle yolks.

Studies examining maternal transfer of toxicants in amphibians have been limited. Grillitsch and Chovanev (1995) performed a field study in which they measured the concentrations of heavy metals and pesticides in anuran spawn, tadpoles, water, and sediment. Spawn, as defined by the authors, are embryos at Gosner (1960) stage 15 ± 2, (neurula). They found Cd, copper (Cu), lead (Pb) and zinc (Zn) residues in spawn and tadpole samples of *Bufo bufo, Rana dalmatina,* and *Rana ridibunda* at higher levels than sediment concentrations. Although maternal transfer could be a source of metal contamination in spawn, the possibility of water related metal contamination should not be discounted.

We analyzed the Cd content in the fertilized eggs from the mating of control and exposed females to non-exposed males (Table 3). The Cd content was remarkably low, even lower than concentrations found in embryos at 24 h. One possible explanation for this is the method of weighing the fertilized eggs. Fertilized eggs from exposed females were soft and very fragile. While determining wet weight, it is probable that egg mass was lost and therefore the Cd content

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in the fertilized eggs is most likely understated. We have since modified our protocol to include dry weight of the eggs and embryos in the determination of Cd content.

We also analyzed the Cd concentration in progeny from Cd-exposed females. We found that embryo mortality from females exposed to 0.75 mg/kg was significantly greater than controls (Table 1). However, embryos from Cd-exposed females contained nearly identical Cd concentrations as embryos from control females (Table 3). It may be possible that the embryos that died contained a higher concentration of Cd than the survivors. Embryos from control females exhibited Cd concentrations that were very similar to the concentrations in embryos from females exposed at the lowest level in a similar study (Kotyzova and Sunderman 1998). However, Kotyzova and Sunderman (1998) exposed female frogs to Cd dermally, stimulated ovulation with hCG, then fertilized the eggs *in vitro*, warranting caution when comparing results. Interestingly, the Cd content in the embryos decreased over the duration of the FETAX assay. In order to determine if the embryos were eliminating Cd over the duration of the FETAX assay, the solution in the dishes containing the embryos was sampled every 24 h and analyzed for Cd. The Cd concentration of the FETAX solution remained constant, barely above detection limits, throughout the assay (results not shown). It is possible that the Cd in the solution is too diluted to detect. Therefore, the following warrants further investigation: 1) why is measurable Cd present in the embryos from control females and 2) what is the mechanism for embryonic Cd decline over the course of the FETAX assay?

We have found a significant increase in embryo malformations from females exposed at 0.75 mg/kg. The malformations found in the progeny in the present study were similar to those found in direct FETAX exposure studies of *Xenopus* embryos to Cd (Sunderman et al., 1991; Herkovits, et al., 1997) and in embryos from Cd-exposed females (Kotyzova and Sunderman, 1998).

The wet weight of the liver, spleen and ovaries was taken during the necropsy and analyzed in comparison to the body weight (Table 4). Hamada et al. (1998) found that after eight weeks of Cd exposure, the spleen weight of exposed rats doubled over that of controls. In addition, red blood cell density has been shown to increase in Cd-exposed rats, causing them to become anemic (Kunimoto and Miura, 1986). We did not, however, find a significant increase in the spleen-to-body weight ratio in stimulated frogs. Also the ovary-to-body weight and liver-to-body weight ratios were similar in all frogs. These findings are in contrast to data from other

species. Jana and Sahana (1988) found that the liver and ovary weights of the freshwater fish *Claria batarachus* (L.) were decreased and the protein content was increased after Cd exposure. Additionally, a significant decrease in ovary weight was noted after a single s.c. injection of Cd into the Indian koel (*Eudynamys scolopacea*) (Sarkar et al., 1976). Similar results were observed when *Channa punctatus* were exposed to nonlethal levels of mercury (Dey and Bhattacharya, 1989). In the present study, we noted that ovaries from exposed frogs often contained large amounts of fluid in the theca. Therefore, dry weights will be used in future studies in addition we will analyze the protein content of organs, oocytes and embryos.

In non-stimulated frogs, however, there was a statistically significant increase in the spleen-to-body weight ratio in two exposure groups (0.5 mg/kg and 3.0 mg/kg). It may be possible that because the ovaries in non-stimulated frogs were less metabolically active they sequestered less Cd and the Cd concentrated in the spleen instead causing splenomegaly. Support is lent to this hypothesis when the stimulated frogs are examined. There was no statistically significant increase in the spleen-to-body weight ratio in stimulated frogs. The majority of Cd was probably incorporated into the ovaries instead. This hypothesis should be tested by examining the Cd concentration in the spleens of non-stimulated and stimulated female frogs.

In summary, preliminary evaluation of the proposed methods of reproductive assessment appear favorable. We have made numerous improvements to the protocol. The growth rate of oocytes has been determined and from that the ideal length of exposure and of the assay have been resolved. Additionally, the use of hCG-stimulated frogs has been shown to produce the most consistent results. We have found the collagenase technique of liberating oocytes in order to sort and stage them to produce the most accurate results. In order to maintain good statistical power, no less than 200 oocytes should be sorted and staged. Furthermore, the least number of frogs in each treatment group should be seven, however, the larger the sample size the better. Analysis of the Cd content in dry ovaries, spleen, and liver are also informative endpoints.

This study has demonstrated that the stages of oogenesis are sensitive to Cd and that the oocyte population at each stage of oogenesis is a valuable endpoint for a reproductive toxicity assay. Additionally, we have shown that Cd is transferred maternally and that the FETAX assay can be easily modified to demonstrate the effects of maternal exposure to reproductive toxicants upon progeny thereby providing vital information on transgenerational effects. Thus, validation

should proceed when so indicated by the other investigators. In terms of future work, we will complete our assessment of exposure regimes and evaluation of the reproductive toxicity assessment methods described. Further studies are needed to ascertain the source of elevated Cd concentrations in the embryos from control females as well as the disappearance of Cd over the course of the FETAX assay. Additionally, other methods of assessing Cd toxicity to embryos and body organs, such as protein content analysis, should be explored. Once this effort is complete, we can begin the process of method validation and preparation of the protocol into an ASTM standard guide.

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APPENDIX A

TABLES

TABLE 1

Effect of Route of Exposure/Administration on Boron Accumulation in Xenopus - Total Boron Levels

Route[1]	Testes	B[2] Ovaries	Oviducts
Dermal[3]	17.8 (1.1)	40.5 (2.1)	---
Oral[4]	191.3 (12.3)	333.2 (17.3)	---
i.p.	50.9 (3.3)	58.6 (3.0)	---
s.c.	97.2 (6.2)	151.2 (7.8)	---

[1] 100 ug B/Kg/day wet weight administered daily for 5 days.

[2] Expressed as $\mu g/Kg$ wet weight with folds greater than control provided in parentheses.

[3] Exposure provided via culture water (FETAX Solution). Daily renewal for 5 days.

[4] Administered via rat chow. Total B administered = 1600 $\mu g/Kg/day$ for 28 days.

TABLE 2

Impact of Route of Administration on
Boron Accumulation - Percent of Dose Administered

Route[1]	Testes	B^2 Ovaries	Oviducts
Dermal[3]	3.6	8.1	---
Oral[4]	1.0	1.7	---
i.p.	10.2	11.7	---
s.c.	19.4	30.2	---

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[1] 100 ug B/Kg/day wet weight administered daily for 5 days.

[2] Expressed as µg/Kg wet weight

[3] Exposure provided via culture water (FETAX Solution). Daily renewal for 5 days.

[4] Administered via rat chow. Total B administered = 1600 µg/Kg/day for 28 days.

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TABLE 3

Effect of Route of Exposure/Administration on
Cadmium Accumulation in Xenopus - Total Cadmium Levels

Route[1]	Testes	Cd[2] Ovaries	Oviducts
Dermal[3]	10.0 (1.1)	42.8 (1.5)	---
Oral[4]	0.0 (0.8)	281.7 (10.0)	---
i.p.	3823 (2.4)	5851.7 (206.7)	---
s.c.	3453.0 (2.2)	6461.7 (228.3)	7001.7 (247.4)

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[1] 2.5 mg Cd/Kg wet weight administered daily for 5 days.

[2] Expressed as μg/Kg wet weight with folds greater than control provided in parentheses

[3] Exposure provided via culture water (FETAX Solution). Daily renewal for 5 days.

[4] Administered via earthworms laced with Cd. Total Cd administered = 2.5 mg/Kg wet weight/day.

TABLE 4

Impact of Route of Administration on
Cadmium Accumulation - Percent of Dose Administered

Route[1]	Testes	B[2] Ovaries	Oviducts
Dermal[3]	0.08	0.3	---
Oral[4]	0.0	2.3	---
i.p.	30.6	46.8	---
s.c.	27.6	51.7	56.0

[1] 2.5 mg Cd/Kg wet weight administered daily for 5 days.
[2] Expressed as μg/Kg wet weight with folds greater than control provided in parentheses
[3] Exposure provided via culture water (FETAX Solution). Daily renewal for 5 days.
[4] Administered via earthworms laced with Cd. Total Cd administered = 2.5 mg/Kg wet weight/day.

TABLE 5

Results of Modified FETAX Toxicity Testing on Progeny of Cd-exposed and control female *Xenopus laevis*.   (* indicates $p < 0.05$)

** Exposure group (mg/kg)	Mortality (%) & number of frogs	Malformation (%) & number of frogs	Embryo length (mm) mean (sd) & number of embryos
control	8.8 (n=11)	1.63 (n=11)	9.18 ($\pm$0.036) (n=2044)
0.50	50.0 (n=1)	59.3 (n=1)	8.01 ($\pm$0.042) (n=15)
0.75	*40.6 (n=4)	*8.91 (n=4)	8.50 ($\pm$0.072) (n=404)
1.00	13.5 (n=4)	3.52 (n=4)	8.96 ($\pm$0.083) (n=597)

** frogs exposed at 3.0 mg/kg did not breed

TABLE 6

Mean (sd) Cd concentration in the ovaries of non-stimulated and stimulated frogs and the percentage of Cd injected into frog that was accumulated by the ovary. (* indicates $p < 0.05$)

	Exposure Group mg/kg	Cd Concentration (ug/g wet weight)	% Cd injected that ovary accum.	n (# frogs)
n-stimulat frogs	control	0.059 (±0.109)	no Cd injected	7
	0.5	0.648 (±0.288)	3.107 (±1.645)	6
	5	*1.530 (±\0.292)	0.737 (±0.209)	6
Stimulate frogs	control	0.068 (±0.057)	no Cd injected	3
	0.5	*9.176 (±2.926)	22.21 (±7.21)	8
	0.75	5.017 (±2.662)	10.08 (±5.14)	6
	1.00	6.400 (±3.091)	9.13 (±3.13)	8
	3.00	*9.230 (±3.144)	5.79 (±2.17)	14

TABLE 7

Mean (sd) Cd concentration present in fertilized eggs and embryos of stimulated frogs. Eggs and embryos were collected and analyzed on the basis of weight.

Exposure Group	Cd. conc in fertilized	Cd. concentration in embryos (ug/g wet weight)				
mg/kg	eggs (ug/g wet weight)	24 h	48 h	72 h	96 h
control	0.057 (±0.035)	0.467 (±0.035)	0.365 (±0.127)	0.126 (±0.035)	0.058 (±0.060)
0.75	0.543 (±0.670)	0.929 (0)	0.879 (±0.103)	1.866 (±2.411)	0.089 (0)
1.00	0.505 (±0.203)	0.722 (±0.417)	1.135 (0.658)	0.551 (±0.523)	0.224 (±0.243)

TABLE 8

Mean (sd) organ:body weight ratios in non-stimulated and stimulated frogs. (Oneway ANOVA and Dummett's Tests)
* indicates P<0.0001

	Number	Exposure Group (mg/kg)	Liver (g)	Spleen (g)	Ovary (g)
Non-stimulated frogs	14	control	0.042 (± 0.150)	0.001 (± 0.001)	0.134 (± 0.033)
	7	0.5	0.044 (± 0.010)	*0.003 (± 0.001)	0.152 (± 0.025)
	7	3.0	0.045 (± 0.005)	*0.007 (± 0.015)	0.131 (± 0.027)
	14	5.0	0.041 (± 0.007)	0.001 (± 0.000)	0.139 (± 0.030)
Stimulated frogs	3	control	0.034 (± 0.005)	0.001 (± 0.001)	0.105 (± 0.028)
	8	0.5	0.109 (± 0.151)	0.003 (± 0.002)	0.077 (± 0.032)
	6	0.75	0.042 (± 0.005)	0.001 (± 0.001)	0.109 (± 0.055)
	8	1.0	0.040 (± 0.008)	0.003 (± 0.004)	0.143 (± 0.061)
	14	3.0	0.0449 (± 0.013)	0.003 (± 0.002)	0.086 (± 0.040)
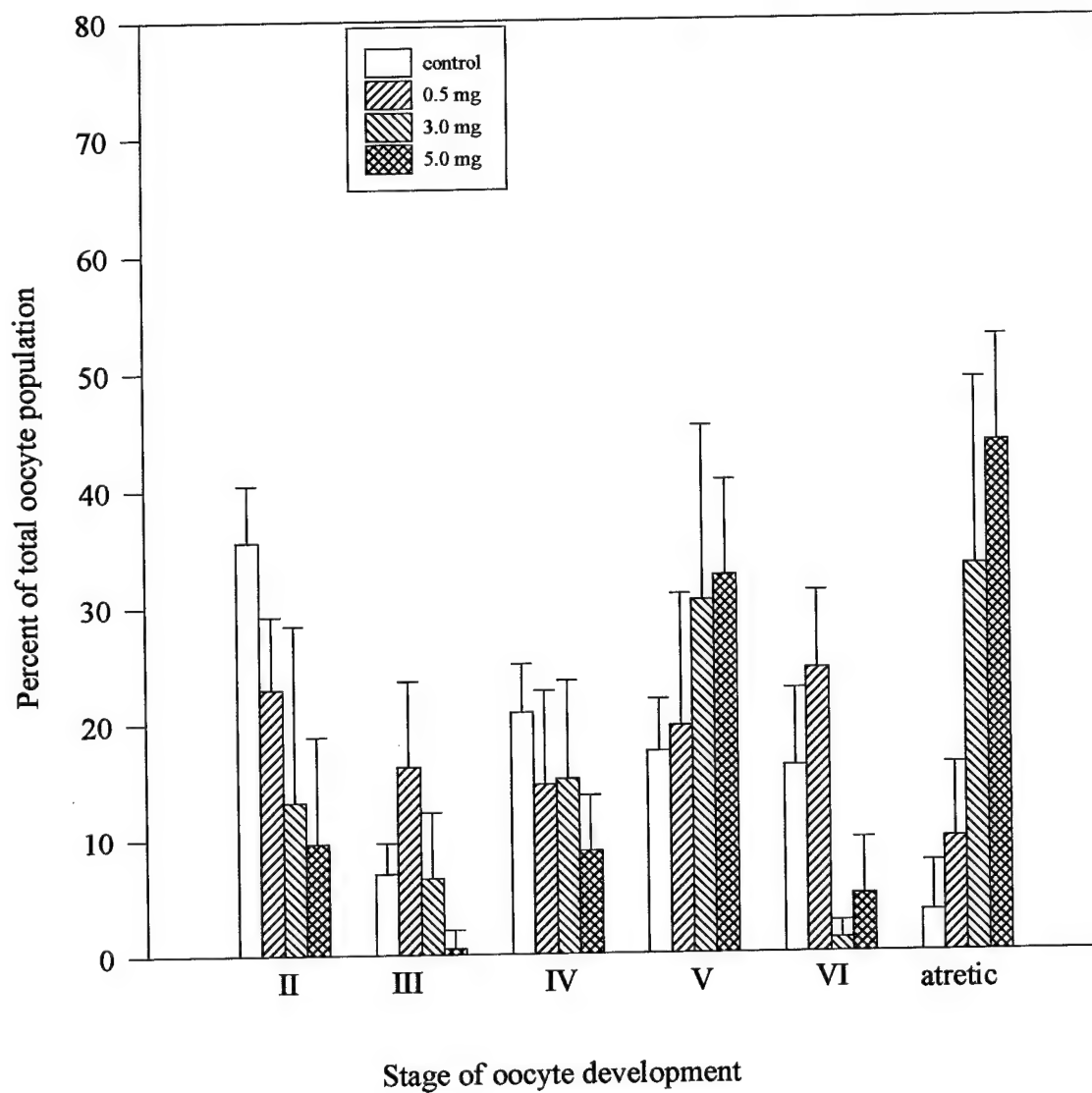
Figure 1. Mean percentage of oocytes present at each stage of oogenesis in non-stimulated frogs. Error bars indicate standard deviation.
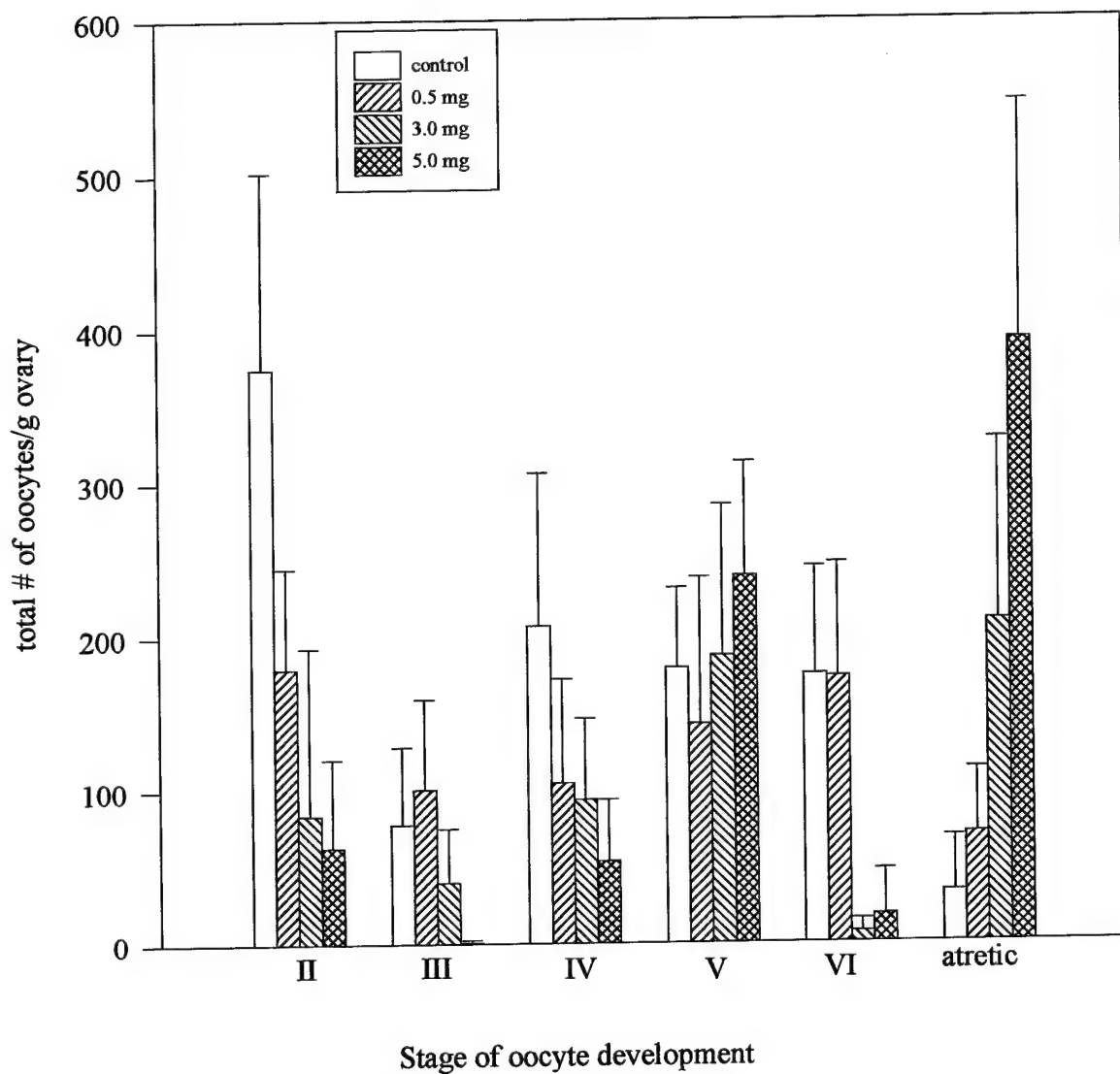
Figure 2. Mean number of total oocytes/g ovary present at each stage of oogenesis in hCG-non-stimulated frogs. Error bars represent standard deviation.
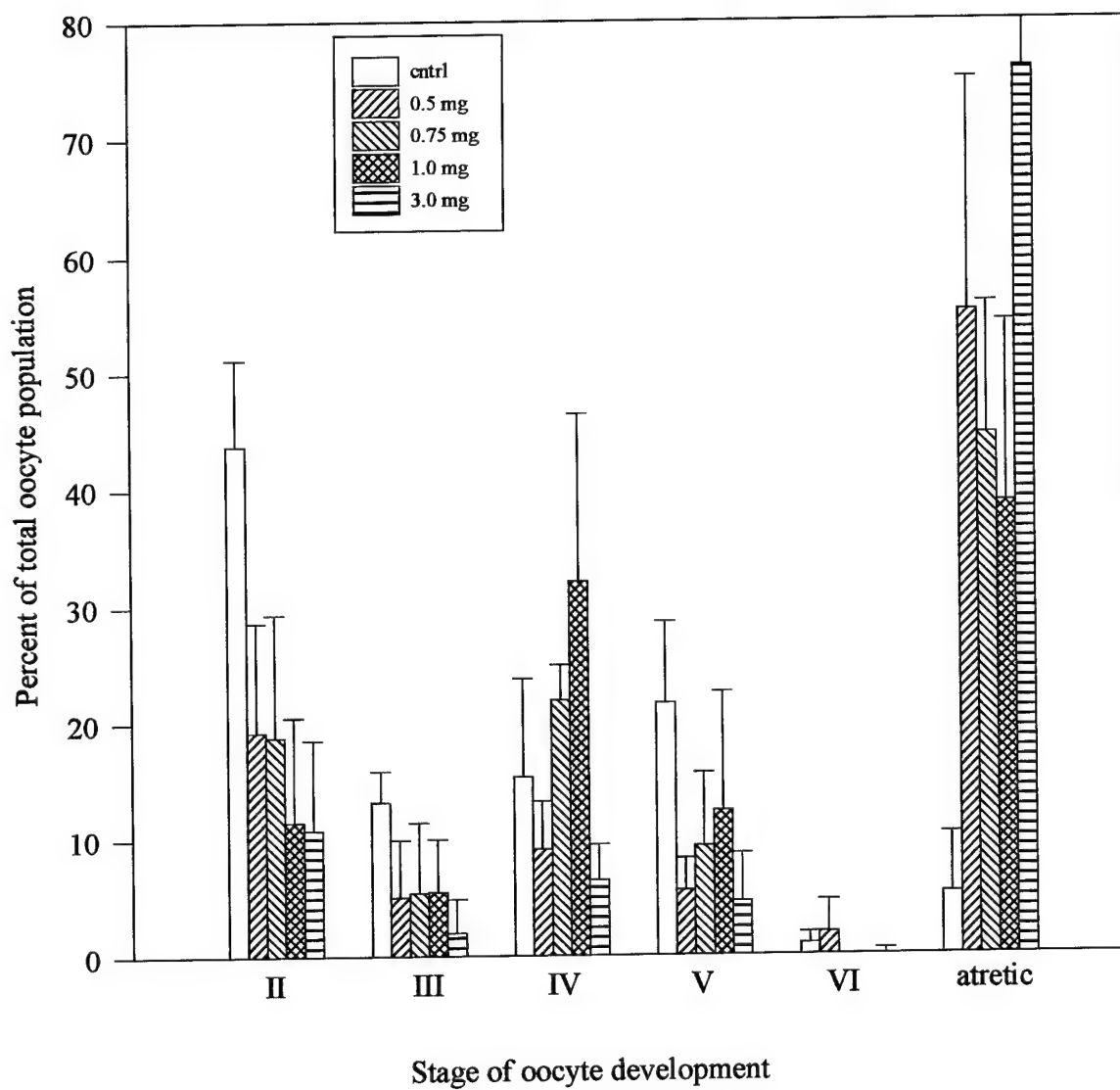
Figure 3. Mean percentage of oocytes present at each stage of oogenesis
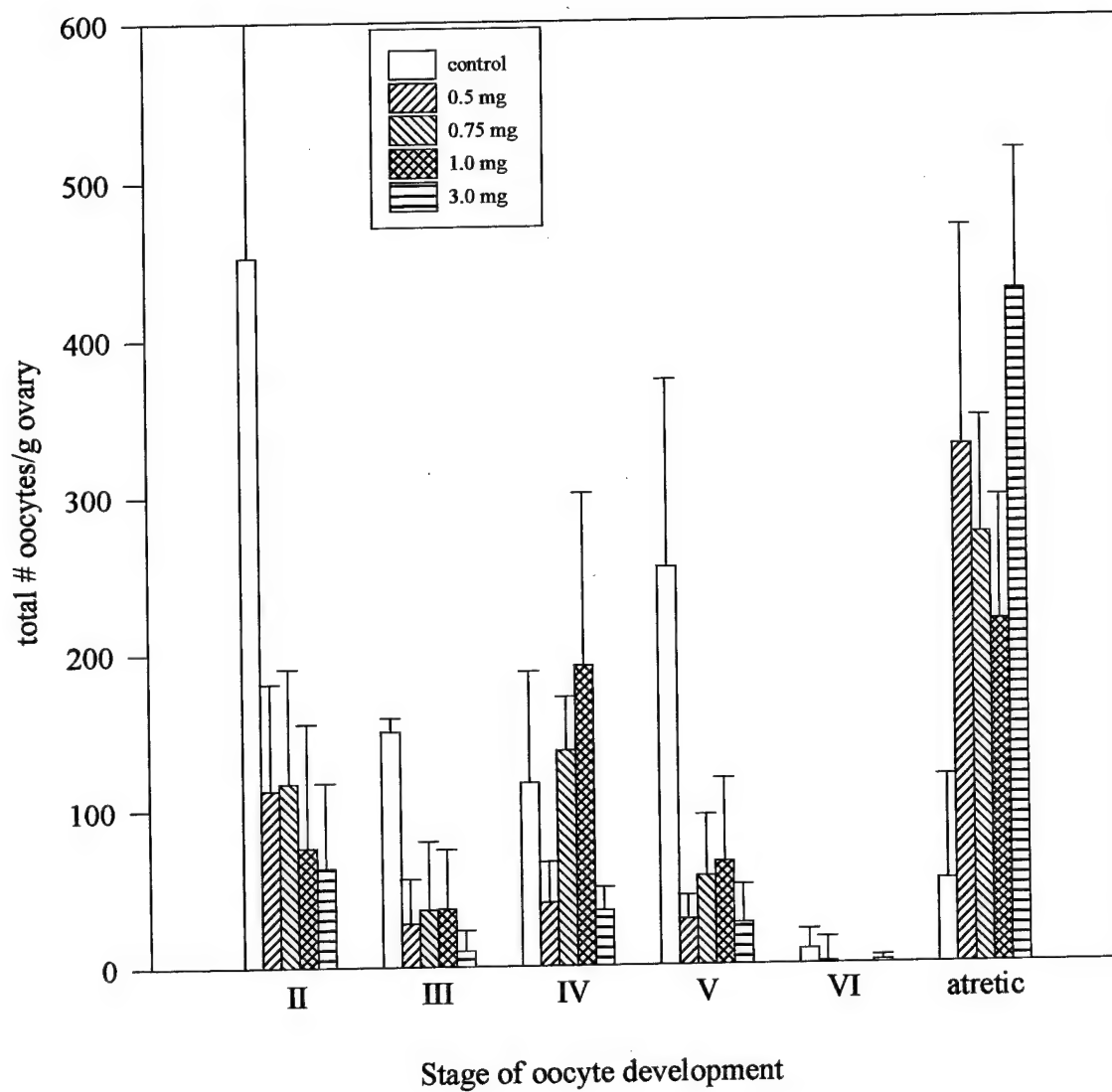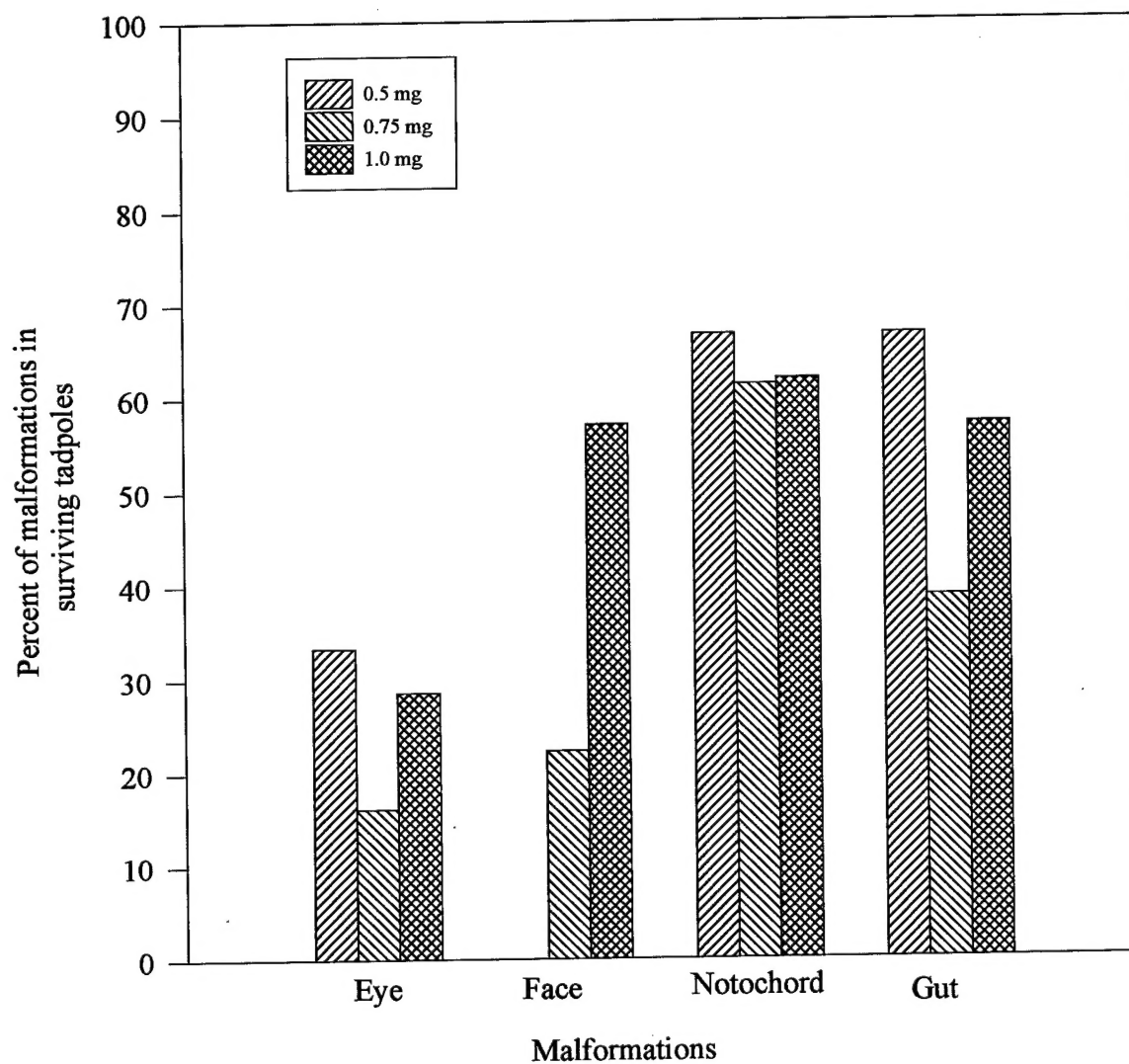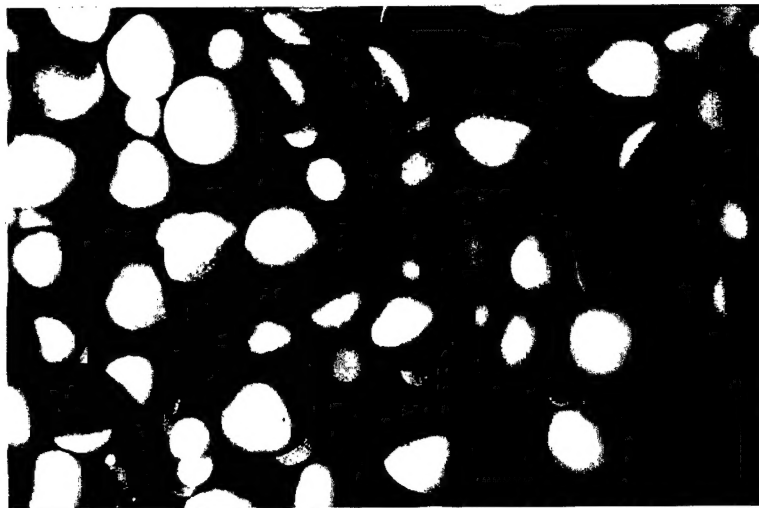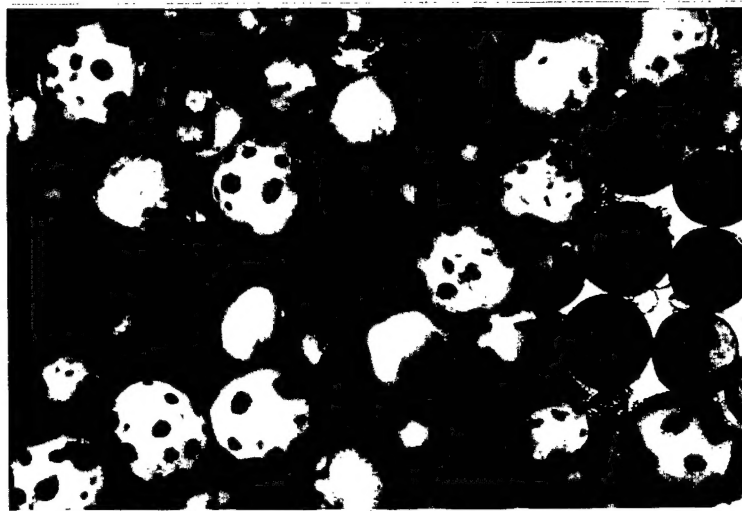in hCG-stimulated frogs. Error bars represent standard deviation.

Figure 4. Mean number of total oocytes/g ovary present at each stage of oogenesis in hCG-stimulated frogs. Errors bars represent standard deviation.

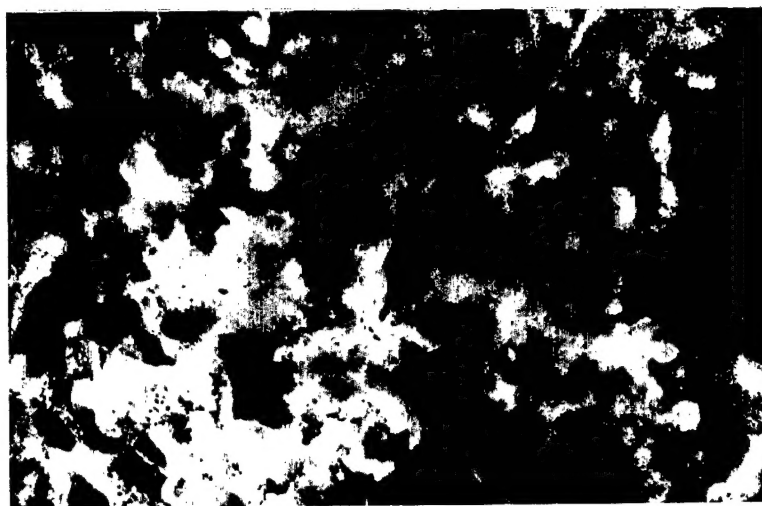Figure 5. Incidence of malformations in tadpoles after maternal exposure to Cd.

Figure 6. Oocytes from (A) control female in which stage II - VI oocytes are clearly visible, (B) from female exposed at 0.5 mg/kg (noticeable change in morphology), (C) from female exposed at 3.0 mg/kg (majority of oocytes are atretic and completely degenerated.

Figure 7. Notochord malformations. (A) embryo from control female. (B) and (C) embryos from female exposed at 0.75 mg/kg.
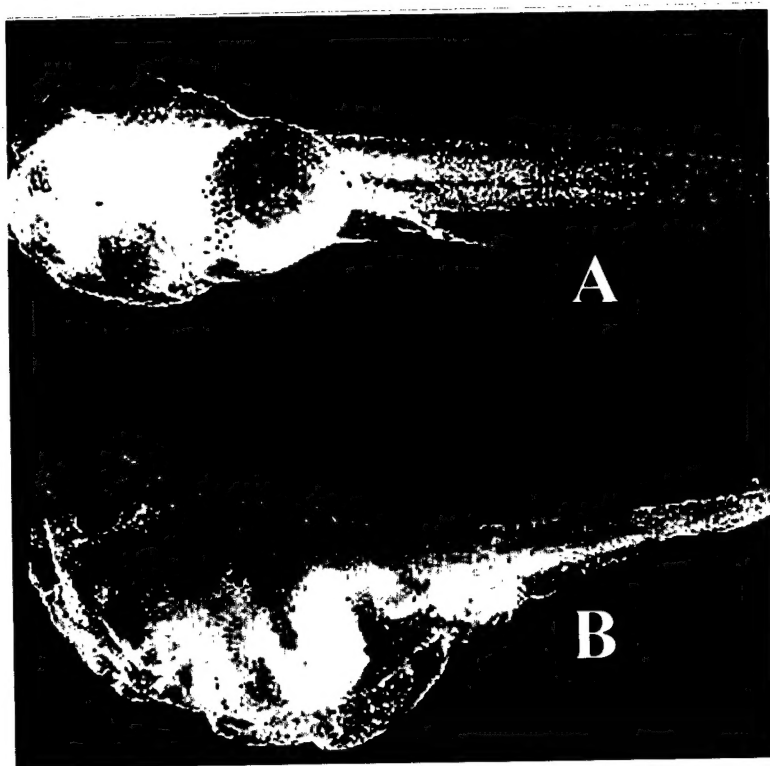
Figure 8. Gut malformations. (A) embryo from control female.
(B) embryo from female exposed at 0.75 mg/kg.